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by the Nonhomologous CP77 Gene

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We investigated the host-range restriction of a vaccinia virus (VV) K1L deletion mutant in rabbit kidney RK13 cells and the ability of the nonhomologous cowpox virus CP77 gene to overcome this block. Viral early mRNAs were made by K1L$^-$ VV but early protein synthesis was arrested consistent with a translational block. Replication of viral DNA did not occur and neither intermediate nor late mRNAs or proteins were detected. These results indicated that host-range restriction occurs earlier in RK13 cells than in Chinese hamster ovary cells (CHO) cells infected with CP77$^-$ VV, where the block occurs at translation of intermediate stage mRNA. We confirmed a report (Perkus *et al., Virology* 179, 276–286, 1990) that the CP77 gene, which allows VV replication in CHO cells, could replace the K1L gene for plaque formation in RK13 cells. However, the size of the plaques formed by K1L$^-$CP77$^+$ VV was smaller than those formed by K1L$^+$CP77$^-$ VV. Single-step growth curves also showed that the CP77 gene could functionally replace the K1L gene, although formation of infectious virus was delayed and did not reach the same level as that of K1L$^+$ VV. Most surprisingly, the dramatic shutoff of viral and host gene expression was similar in RK13 cells infected with K1L$^-$CP77$^-$ VV and K1L$^-$CP77$^+$ VV and little difference was noted for the first 6 hr. Subsequently, in cells infected with the K1L$^-$CP77$^+$ VV, viral early protein synthesis was spontaneously resurrected and the replication cycle proceeded. Despite the absence of homology, K1L and CP77 gene products appear to be acting in a common virus/cell interaction pathway.

INTRODUCTION

Although Orthopoxviruses can infect a wide variety of mammalian and avian cells, there are host-range mutants that fail to replicate in specific lines derived from pig kidney (Fenner and Sambrook, 1966; Lake and Cooper, 1980; Moyer *et al.,* 1980; Moyer and Graves, 1982), Chinese hamster ovary (CHO) (Drillien *et al.,* 1978; Njayou *et al.,* 1982), or human sources (Drillien *et al.,* 1981; Meyer *et al.,* 1991; Perkus *et al.,* 1990). These mutants provide a unique opportunity to investigate specific virus/host interactions. The CHO host-range defect of laboratory strains of vaccinia virus (VV) can be complemented by the cowpox CHO *hr* gene, also called CP77 (Spehner *et al.,* 1988). VV has a CP77 homolog but the open reading frame is interrupted in strain WR (Kotwal and Moss, 1988) and deleted in strain Copenhagen (Goebel *et al.,* 1990). A spontaneous human host-range mutant VV, isolated by Drillien *et al.* (1981), contains a large deletion near the left end of the genome. Marker transfer experiments indicated that the human host-range defect could be overcome by insertion of the K1L gene, one of several open reading frames contained within the deleted DNA segment (Gillard *et al.,* 1985, 1986). Subsequently, Perkus *et al.* (1990) discovered a functionally equivalent human host-range gene, C7L, within the same segment of deleted DNA but further showed that K1L was specifically required for replication in rabbit kidney (RK13) cells. Deletions of other Orthopoxvirus genes have produced host-specific effects that will not be considered here (Ali *et al.,* 1994; Brooks *et al.,* 1995; Takahashi-Nishimaki *et al.,* 1991).

Initial host-range studies with the Copenhagen strain of VV in CHO cells demonstrated a block at an early stage of the replication cycle accompanied by rapid inhibition of host and viral protein synthesis (Drillien *et al.,* 1978; Njayou *et al.,* 1982). More recent investigations with the WR strain of VV indicated that synthesis of vaccinia viral early mRNA and proteins, DNA, and intermediate stage mRNAs occurred but neither intermediate viral proteins nor late mRNAs or proteins were made in CHO cells (Ramsey-Ewing and Moss, 1995). Ink *et al.* (1995) reported that apoptosis occurred in CHO cells infected with VV or VV recombinants expressing the cowpox CP77 gene but was delayed under the latter conditions. Relationships between apoptosis, inhibition of viral intermediate protein synthesis and host restriction remain to be determined, since only a minority of cells appear to undergo this type of cell death (Ramsey-Ewing, unpublished).

Drillien *et al.* (1981) reported that the VV mutant with the large deletion encompassing both K1L and C7L was blocked at an early stage of replication in human cells: cytoplasmic RNA synthesis and viral protein synthesis occurred transiently and cytoplasmic DNA synthesis was

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greatly reduced. Both ectromelia virus and vaccinia virus with K1L deletions exhibited transient synthesis of viral proteins in RK13 cells (Chen *et al.,* 1993; Sutter *et al.,* 1994). Using the modified vaccinia virus Ankara (MVA) strain which has a truncated K1L gene (Meyer *et al.,* 1991), Sutter *et al.* (1994) noted that viral DNA replication was blocked and that intermediate mRNAs were not made in RK13 cells, suggesting a difference from the host restriction phenotype obtained with the WR strain of VV in CHO cells. Although the MVA phenotype in RK13 cells was reversed by expression of the K1L gene, MVA has multiple deletions and remains host restricted for other mammalian cells lines even after restoration of K1L, invalidating direct comparisons with more standard VV strains such as WR or Copenhagen.

Perhaps the most remarkable feature of the host-range genes CP77, K1L, and C7L is their apparent functional equivalence despite the absence of any evident sequence similarities. Perkus *et al.* (1990) discovered that: (i) C7L is functionally equivalent to K1L for replication in human cells; (ii) C7L can be distinguished from K1L by its inability to replace K1L for replication in RK13 cells; (iii) CP77 can substitute for K1L in RK13 cells; and (iv) K1L, C7L, or CP77 allows replication of VV in pig kidney cells. The efficiency of complementation, however, cannot be deduced from this initial report, since only the plaque titers in various cell lines were reported with no information regarding plaque sizes or virus yields. Virus yield experiments of Oguiura *et al.* (1993) confirmed that K1L and C7L were functionally equivalent in human cells, although in some lines the $K1L^+C7L^-$ gave higher yields than the $K1L^-C7L^+$. They also confirmed that C7L could not replace K1L in RK13 cells and discovered that both K1L and C7L were required in rat NRK cells and that C7L was specifically required in hamster Dede cells. The functional relationship of CP77 with the other host-range gene, that is, K1L was not evaluated in the latter study.

We considered that further experimental work was needed to more precisely determine the stage at which a specific $K1L^-$ VV mutant is blocked in RK13 cells and the ability of the CP77 gene to relieve this defect. In the present investigations, we compared viral replication and gene expression in RK13 cells infected with recombinant VV (strain WR) that contained reporter genes and were $K1L^+CP77^-$ (wild type), $K1L^-CP77^-$, or $K1L^-CP77^+$. A preliminary report of this work was presented at the 14th Annual Meeting of the American Society for Virology, 1995.

MATERIALS AND METHODS

Cells and viruses

BS-C-1 cells (kidney, African green monkey) and CV-1 (kidney, African green monkey) cells were grown in minimum essential medium (MEM) supplemented with 2.5% fetal calf serum (FCS). For guanine phosphoribosyltransferase (*gpt*) selection, viruses were isolated by plaquing on BS-C-1 monolayer cultures in the presence of 25 μg/ml mycophenolic acid (MPA), 250 μg/ml xanthine, and 15 μg/ml hypoxanthine as described (Falkner and Moss, 1988). RK13 cells were grown in MEM supplemented with 10% FCS. CHO cells were grown in Ham's F-12 medium supplemented with 5% FCS. $HuTK^-$ 143 (osteosarcoma, human) cells were grown in MEM supplemented with 10% FCS and recombinant viruses selected in the presence of 25 μg of 5-bromodeoxyuridine per milliliter. VV strain WR and recombinants thereof were propagated and plaque assayed as described (Earl and Moss, 1991).

Recombinant viruses

Protocols for construction of recombinant VV by homologous recombination at the thymidine kinase (*tk*) locus have been published (Earl and Moss, 1991). For recombinant viruses in this study, we used a dual reporter plasmid (pREβCAT) with TK flanking sequences in which the *cat* gene, encoding chloramphenicol acetyltransferase (CAT), is regulated by a viral early promoter (virus growth factor, VGF), and the *lacZ* gene, encoding β-galactosidase (βGAL), is regulated by a viral late promoter (11K structural protein) (Ramsey-Ewing and Moss, 1995). The pREβCAT plasmid was then coprecipitated with VV WR viral DNA and transfected into VV WR-infected CV-1 cells producing the recombinant virus $vK1L^+CP77^-$ in which the dual reporter cassette is recombined into the *tk* locus (Fig. 1). To prepare the $K1L^-$ knockout virus, we first reassembled the K1L gene of VV strain WR from two plasmids obtained from M. Merchlinsky, p*Hin*dIIIK and p*Hin*dIIIM, that contain the *Hin*dIII K and M fragments of VV strain WR, respectively. Plasmid p*Hin*dIIIK was digested with *Hin*dIII and *Eco*RV and the resultant 847-bp fragment containing the 5′ end of the K1L gene was isolated. Plasmid p*Hin*dIIIM was digested with *Hin*dIII and *Sph*I and the resultant 1506-bp fragment containing the coding region and 3′ end of the K1L gene was isolated. The K and M fragments were both subcloned into pUC19 that had been linearized with *Sma*I and *Sph*I. The resultant plasmid, pREK1L, was digested with *Bgl*II and recircularized to produce plasmid $pREK1L^-$, which contains a K1L gene from which the major portion of the coding region (amino acids 1 thru 253) is deleted. A *Bgl*II–*gpt*–*Bgl*II cassette, in which the *Escherichia coli gpt* gene is regulated by the VV early promoter (7.5K) and followed by a VV early termination signal, was prepared in a recombinant PCR reaction using plasmid pGEMgpt (Blasco *et al.,* 1991) as template. The isolated fragment was digested with *Bgl*II and ligated to $pREK1L^-$ that had been linearized with *Bgl*II to produce plasmid pK1Lgpt. To contruct recombinant virus $vK1L^-CP77^-$, plasmid pK1Lgpt was used as a donor in homologous recombination with $vK1L^+CP77^-$. The resultant recombi-

nant virus, vK1L$^-$CP77$^-$, was isolated as a TK$^-$ βGAL$^+$ MPA-resistant plaque. To construct the vK1L$^+$CP77$^+$ and vK1L$^-$CP77$^+$ recombinant viruses, we first subcloned the 2.3-kb *Eco*RI/*Pst*I fragment derived from pEA36 (Spehner *et al.,* 1988), which contains the entire CHO *hr* gene encoding the CP77 protein, into the *Eco*RI/*Pst*I sites of pUC19. The resultant plasmid, pRECP77, was used as a donor in homologous recombination with either vK1L$^+$CP77$^-$ or vK1L$^-$CP77$^-$ to create vK1L$^+$CP77$^+$ (Ramsey-Ewing and Moss, 1995) and vK1L$^-$CP77$^+$, respectively (Fig. 1). In addition to the dual reporter cassette at the *tk* locus, recombinant viruses vK1L$^+$CP77$^+$ and vK1L$^-$CP77$^+$ have an intact copy of the CHO *hr* gene instead of the disrupted WR copy (Kotwal and Moss, 1988) at the corresponding region of the *Hin*dIII C fragment.

Analysis of viral genome replication

RK13 cells were infected with recombinant viruses at a multiplicity of 10 plaque-forming units (PFU)/cell. At various times, cells were washed twice and scraped in phosphate-buffered saline (PBS). The cell pellet was resuspended in lysis buffer [20 m*M* Tris–HCl (pH 8.0), 10 m*M* EDTA, 0.75% sodium dodecyl sulfate (SDS), 570 mg/ml proteinase K] and viral DNA was isolated by phenol/chloroform extraction and ethanol precipitation as described (Earl and Moss, 1991). Viral DNA samples were applied to a nylon filter using a slot blot apparatus as described by the manufacturer (Hoefer) and hybridized to *cat* DNA sequences that had been labeled by random oligonucleotide priming as specified by the manufacturer (Promega).

Analysis of reporter gene expression

Cells were infected with recombinant viruses at a multiplicity of 30 PFU/cell. At various times after infection, the cells were washed twice with PBS and incubated for 15 min at 37° in 1× reporter lysis buffer (Promega). The lysate was centrifuged and the supernatant retained for further analysis. Protein content of each lysate was determined colorimetrically using the Pierce Coomassie blue reagent. Either equal volumes of lysates or equal amounts of protein were used in βGAL assays as described by the manufacturer (Promega). Standard protein and enzyme activity curves were prepared for quantitative analysis of assay results.

For *in situ* analysis of βGAL expression, RK13 cells were infected with serial dilutions of recombinant virus stocks. At 24 hpi, infected cell monolayers were washed twice with PBS and then fixed in 1% gluteraldehyde in 0.1 *M* sodium phosphate (pH 7.0), 1 m*M* magnesium chloride for 15 min at room temperature. Assay buffer [0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) in 10 m*M* sodium phosphate (pH 7.0), 1 m*M* magnesium chloride, 150 m*M* sodium chloride, 3.3 m*M* potassium ferricyanide, 3.3 m*M* potassium ferricyanide trihydrate] was added and the plates were incubated an additional 12 to 16 hr at 37°. Plates were washed several times and individual representative plaques photographed under PBS.

Analysis of viral protein synthesis

RK13 cells (5×10^5) were seeded into 24-well plates; at 16 to 24 hr, the cells were infected with recombinant viruses at a multiplicity of 30 PFU/cell. From 10 to 20 min before each labeling period, cells were washed twice and incubated in prewarmed medium without methionine. The cells were then incubated in the presence of 75 μCi of [^{35}S]methionine in 0.25 ml of methionine-free medium for 30 min. The labeling medium was removed and the cells were washed twice with ice-cold PBS and then incubated at 37° for 3 to 5 min with hypotonic lysis buffer [20 m*M* Tris–HCl (pH 8.0), 10 m*M* NaCl, 0.5% Nonidet P-40 (NP-40)]. The lysate was collected and centrifuged for 2 min at 12,000 *g* to pellet nuclei. The supernatants containing ^{35}S-labeled polypeptides were stored at −20°. A portion of each sample was mixed with an appropriate volume of SDS/2-mercaptoethanol sample buffer and boiled for 5 min. The samples were resolved by electrophoresis in SDS 10% polyacrylamide gels.

Western blot analysis

Pellets from 10^6 infected cells were incubated with 100 ml lysis buffer [20 m*M* Tris–HCl (pH 7.0), 0.5% Triton X-100 in PBS] for 5 min at 37°. Lysates were centrifuged at 14,000 *g* for 5 min and the supernatants stored at −20°. A portion was mixed with 5× SDS/2-mercaptoethanol sample buffer (5′ to 3′, Inc.), boiled for 5 min, and proteins were resolved in SDS 10% polyacrylamide gels. Proteins were electrotransferred to nitrocellulose membranes and incubated with polyclonal antiserum to vaccinia virus at 1:500 overnight at 4° and then ^{125}I-protein A overnight at 4°.

Immunoprecipitation

Cells were infected with recombinant viruses at a multiplicity of 30 PFU/cell, labeled with [^{35}S]methionine as described above, and harvested at various times. Lysates were prepared in isotonic lysis buffer [50 m*M* Tris–HCl (pH 8.0), 150 m*M* NaCl, 0.02% sodium azide, 0.1% SDS, 100 mg/ml phenylmethylsulfonyl fluoride, 1% NP-40], centrifuged to remove nuclei, and then incubated with polyclonal antiserum to vaccinia virus at a 1:500 dilution at 4° overnight. An equal volume of 20% Protein A–Sepharose beads in PBS was added and incubation continued at room temperature for an additional 2 to 3 hr or at 4° overnight. Immune complexes were washed twice in Triton buffer [300 m*M* NaCl, 50 m*M* Tris–HCl (pH 7.4), 0.1% Triton X-100]. Proteins from immunoprecipitation reactions were recovered by boiling in sample buffer and then

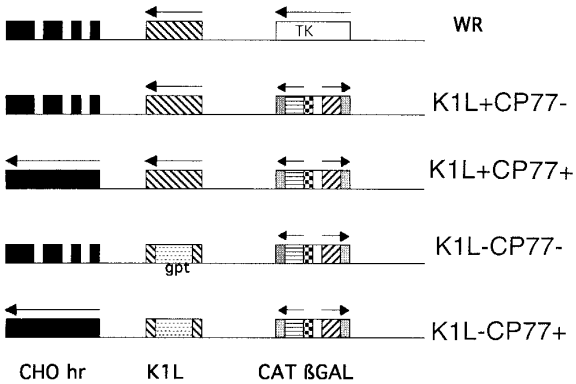

FIG. 1. Genetic features of recombinant vaccinia viruses. The CHO *hr* ORF encoding CP77 is shown as interrupted in parental WR and recombinant K1L$^+$CP77$^-$ and K1L$^-$CP77$^-$ VV and intact in recombinant K1L$^+$CP77$^+$ and K1L$^-$CP77$^+$ VV. The K1L ORF is intact in WR, K1L$^+$CP77$^-$, and K1L$^+$CP77$^+$ and replaced by the *E. coli gpt* cassette in K1L$^-$CP77$^-$ and K1L$^-$CP77$^+$. The arrows show the directions of transcription. All recombinant viruses contain the dual reporter cassette, early-CAT and late-βGAL, at the *tk* locus.

resolved by electrophoresis in SDS 10% polyacrylamide gels.

Preparation and analysis of viral RNA

Viral RNA was isolated from cells that had been infected with recombinant viruses at a multiplicity of 30 PFU/cell. At various times after infection, the cells were washed twice in ice-cold PBS and then pelleted. Approximately 10^6 infected cells were lysed in 100 μl of Direct Protect lysis buffer and aliquots of the lysates added to 50-μl hybridization reactions and processed as specified by the manufacturer (Ambion). Single-stranded ^{32}P-labeled antisense RNA probes (Riboprobes) were prepared by *in vitro* transcription of linearized plasmid or PCR-generated DNA templates as described by the manufacturer (Promega). Probes used in these studies were transcribed from the following templates: CAT and βGAL, plasmids pTRI-cat and pTRI-βGAL (Ambion); and A1L, plasmid pGEM17K (Baldick *et al.,* 1992).

RESULTS

Construction of recombinant vaccinia viruses

To facilitate comparisons, the recombinant viruses used in these studies (Fig. 1) had a common WR genome and contained two reporter genes in the *tk* locus, *cat* and *lacZ* under early (VGF) and late (11K) promoters, respectively. In two viruses, an intact CP77 gene replaced the original interrupted homolog. These CP77$^+$ recombinant viruses were isolated by passage in CHO cells ensuring their functionality; in addition, PCR copies of the gene were sequenced. In two viruses, the K1L gene was almost entirely deleted by insertion of a *gpt* selectable marker. Similar titer stocks of the four recombinant viruses K1L$^+$CP77$^-$, K1L$^+$CP77$^+$, K1L$^-$CP77$^-$, and K1L$^-$CP77$^+$ were prepared in BS-C-1 cells which are permissive for all of them.

Cell-dependent replication of recombinant vaccinia viruses

To examine the functional relationship between the K1L and CP77 genes, we infected BS-C-1 or RK13 cells with individual recombinant viruses and stained the plaques by an *in situ* βGAL assay 24 hr later. The staining assay depends on late expression of the *lacZ* gene and can measure virus spread even in the absence of strong cytopathic effects. The recombinant viruses generated plaques of similar size and staining intensity on BS-C-1 cells indicating that neither K1L nor CP77 was required (Fig. 2). On RK13 cells, however, K1L$^+$CP77$^-$ and K1L$^+$CP77$^+$ gave similar-size stained plaques, K1L$^-$CP77$^-$ gave no plaques, and K1L$^-$CP77$^+$ gave small stained plaques (Fig. 2). In Fig. 2, the photographs of the RK13 cells were enlarged fourfold relative to the BS-C-1 cells in order to better show the small K1L$^-$CP77$^+$ plaques. These results indicated that CP77 compensated for the absence of K1L but that virus spread, as revealed by plaque formation, was inefficient under these conditions.

To quantitate virus replication, RK13 cells were infected with recombinant viruses and the yields were determined at 12, 24, 48, and 72 hr (Fig. 3). The growth curves of K1L$^+$CP77$^-$ and K1L$^+$CP77$^+$ were virtually superimposable, indicating that the CP77 gene does not provide an enhancing effect when the K1L gene is functional. By contrast, the presence of the CP77 gene enhanced the replication of K1L$^-$ virus by 1 to 2 logs (Fig. 3). Replication of K1L$^-$CP77$^+$, however, was delayed and never reached the level of K1L$^+$ viruses. By contrast, the 24-hr yields of K1L$^+$CP77$^-$, K1L$^-$CP77$^-$, and K1L$^-$CP77$^+$ were identical in BS-C-1 cells, indicating the cell-specific nature of the mutations (Table 1). The functionality of the CP77 gene in K1L$^-$CP77$^+$ was demonstrated by a 2- to 3-log increase in virus titer, compared to the corresponding CP77$^-$ virus in CHO cells (Table 1). We concluded that the CP77 gene enhanced replication of a K1L$^-$ VV in RK13 cells but to a lesser extent than in CHO cells.

The effect of host-range genes on cell viability was determined using a trypan blue exclusion assay. The data indicated a correlation between loss of viability and virus replication (Table 1). Most significantly, viability of RK13 cells was less after infection with either K1L$^+$CP77$^-$ or K1L$^-$CP77$^+$ compared to K1L$^-$CP77$^-$. The same correlation held true in CHO cells: viability was less after infection with K1L$^-$CP77$^+$ compared to either K1L$^+$CP77$^-$ or K1L$^-$CP77$^-$.

Viral DNA replication in RK13 cells

VV DNA replication was measured by applying lysates of infected RK13 cells to a membrane which was then

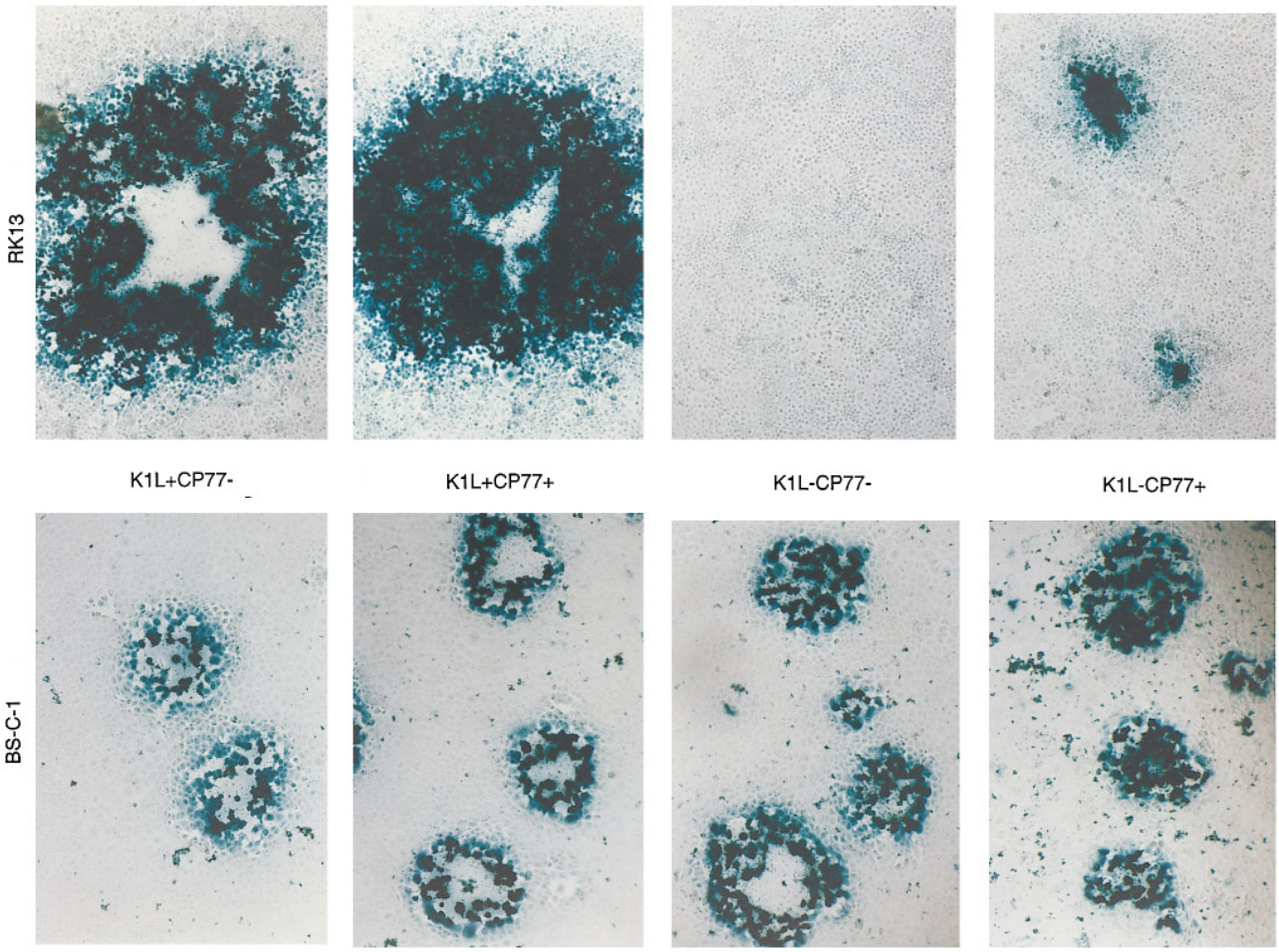

FIG. 2. Plaque formation by recombinant vaccinia viruses. Permissive (BS-C-1) or nonpermissive (RK13) cell monolayers were infected with recombinant K1L$^+$CP77$^-$, K1L$^-$CP77$^-$, K1L$^+$CP77$^+$, or K1L$^-$CP77$^+$ VV. After 24 hr, virus-infected cells were visualized by *in situ* βGAL staining. The photograph of the RK13 cells is enlarged 4$\times$ relative to that of the BS-C-1 cells.

hybridized with a labeled DNA probe. In cells infected with K1L$^+$CP77$^-$ or K1L$^+$CP77$^+$ VV, the amount of viral DNA increased within 4 hr and thereafter (Fig. 4). In the case of K1L$^-$CP77$^-$, viral DNA was not above the 0 time level except perhaps for a small increase at 48 hr. In cells infected with K1L$^-$CP77$^+$ VV, viral DNA steadily increased after a lag of 8 to 16 hr. Both the lag period and the recovery of less viral DNA in the case of K1L$^-$CP77$^+$ VV than K1L$^+$CP77$^-$ or K1L$^+$CP77$^+$ VV were consistent with the virus yield experiment. Control experiments demonstrated that the K1L gene was not required for DNA replication in CHO cells (data not shown).

A lag in DNA replication could arise from a delay in uncoating of the viral genome to provide the template or in formation of the replication apparatus. To distinguish between these alternatives, we took advantage of previous investigations that had demonstrated non-sequence-specific replication of transfected plasmid DNA by viral enzymes in VV-infected cells (DeLange and McFadden, 1986; Merchlinsky and Moss, 1988). The assay depends on the stringent preference of the *Dpn*I restriction endonuclease for the methylated input plasmid DNA; replication is revealed by the accumulation of high-molecular-weight *Dpn*I-resistant DNA. High-molecular-weight *Dpn*I-resistant DNA accumulated between 4 and 8 hr after infection of RK13 cells with wild-type K1L$^+$CP77$^-$ VV (Fig. 5). The absence of such DNA after infection with K1L$^-$CP77$^-$ VV indicated a global replicative block. Significantly, *Dpn*I-resistant DNA was synthesized between 12 and 24 hr after infection with K1L$^-$CP77$^+$ VV. The similar time of onset of replication of the viral genome and transfected plasmid suggested a delay in production of the replication apparatus rather than (or in addition to) uncoating of the viral genome.

Viral RNA synthesis in RK13 cells

VV gene expression can be divided into early, late, and intermediate phases regulated by stage-specific promoters and transcription factors (Moss *et al.*, 1991). The viral DNA brought into the cell to initiate infection serves as the template for early transcription

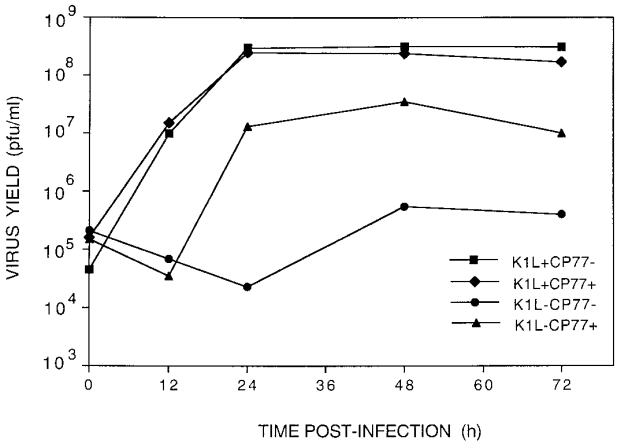

FIG. 3. One-step virus growth curves. RK13 cells were infected with recombinant K1L$^+$CP77$^-$, K1L$^+$CP77$^+$, K1L$^-$CP77$^-$, or K1L$^-$CP77$^+$ VV at a multiplicity of 1 PFU/cell. At the indicated times after infection, cells were harvested and yields of infectious virus determined by plaquing on BS-C-1 cells.

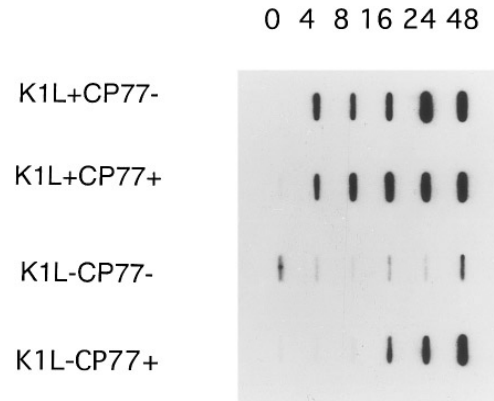

FIG. 4. Viral genome replication. RK13 cells were infected with recombinant K1L$^+$CP77$^-$, K1L$^-$CP77$^-$, K1L$^+$CP77$^+$, and K1L$^-$CP77$^+$ VV at a multiplicity of 10 PFU/cell. Viral DNA was isolated from cells harvested at the indicated hour after infection, immobilized on a nylon membrane, and hybridized to a ^{32}P-labeled DNA probe complementary to the *cat* gene. An autoradiogram is shown.

prior to uncoating, whereas replicated viral DNA is the template for intermediate and late transcription. RK13 cells were infected with recombinant VV and steady-state RNA levels were determined by nuclease protection assays. The *cat* gene, regulated by the early VGF promoter, was chosen to measure early mRNA. Accordingly, a ^{32}P-labeled antisense CAT RNA probe was prepared. The nuclease protection data indicated that CAT RNA peaked at 2 hr after infection then dramatically declined in wild-type (K1L$^+$CP77$^-$) VV-infected cells (Fig. 6). The reappearance of CAT RNA at late times is probably due to read-through late transcription, rather than virus spread to uninfected cells, since the virus multiplicity was 30. In cells infected with K1L$^-$CP77$^-$ VV, CAT RNA levels were also detected at 2 hr but persisted until 6 hr before declining, suggesting delayed shutoff of early transcription. The failure of CAT transcripts to reappear at late times can be attributed to a block in late transcription. A pattern between that of K1L$^-$CP77$^-$ and K1L$^+$CP77$^-$ was seen in cells infected with K1L$^-$CP77$^+$; CAT RNA persisted for 3 hr, declined, and then reappeared at late times.

We used an antisense RNA probe to the intermediate A1L gene (Keck *et al.,* 1990) to measure intermediate transcripts. A1L RNA was detected at 4 hr after infection during wild-type (K1L$^+$CP77$^-$) VV infection of RK13 cells and remained at high levels (Fig. 6), in contrast to the more rapid decline previously seen in synchronously infected HeLa suspension cells (Baldick and Moss, 1993). During infection with K1L$^-$CP77$^-$ VV, only a minute amount of A1L mRNA was detected at 24 hr. By contrast, in RK13 cells infected with K1L$^-$CP77$^+$, A1L RNA was readily detected at 12 hr and increased in amount at later times (Fig. 6). The delay in intermediate mRNA, compared to K1L$^+$CP77$^-$, follows the difference in kinetics of DNA replication.

The *lacZ* reporter gene, regulated by the VV 11K promoter was used to measure late transcription. Late βGAL transcripts were present between 6 and 24 hr after wild-type (K1L$^+$CP77$^-$) infection of RK13 cells, but were not detected during infection with K1L$^-$CP77$^-$ (Fig. 6). In RK13 cells infected with K1L$^-$CP77$^+$, βGAL RNA accumulated between 12 and 24 hpi.

TABLE 1

Virus Growth and Cell Viability

	RK13		CHO		BS-C-1	
Virus	Yield[a]	Viability[b]	Yield	Viability	Yield	Viability
K1L + CP77−	7×10^8	22	5×10^5	77	2×10^8	10
K1L + CP77+	—	25	—	3	—	9
K1L − CP77−	9×10^3	68	3×10^5	73	2×10^8	11
K1L − CP77+	2×10^5	24	1×10^8	25	2×10^8	9

[a] Virus yield after 24 hr (PFU/ml).
[b] Cell viability after 24 hr (percentage).

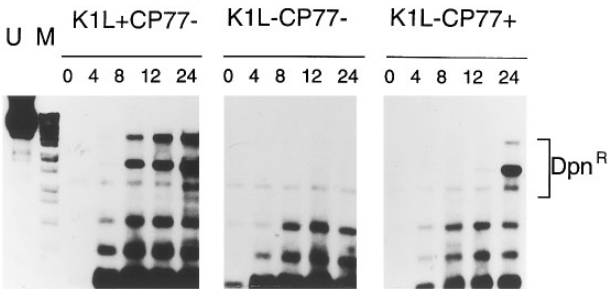

FIG. 5. Plasmid replication. RK13 cells were infected with recombinant K1L$^+$CP77$^-$, K1L$^-$CP77$^-$, or K1L$^-$CP77$^+$ VV at a multiplicity of 10 PFU/cell and then transfected with plasmid pSG5 (Invitrogen). At the indicated times after infection, cytoplasmic DNA was isolated and digested with *Hin*dIII and *Dpn*I. After electrophoretic separation, samples were tranferred to a nylon membrane and hybridized with ^{32}P-labeled linearized pSG5. An autoradiogram is shown. U, uninfected; M, ^{35}S-labeled DNA size markers; Dpn^R, DNA resistant to cleavage with *Dpn*I.

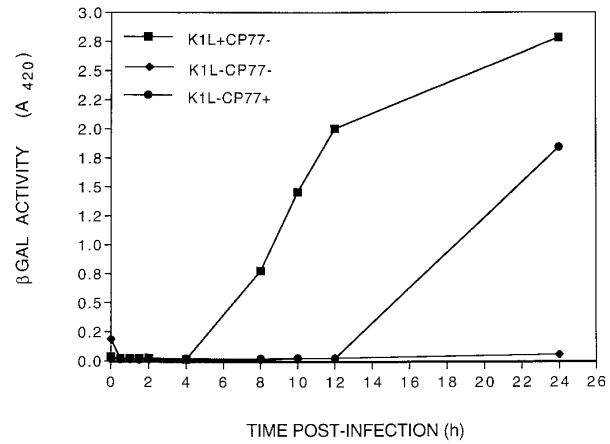

FIG. 7. Late reporter gene expression. RK13 cells were infected with K1L$^+$CP77, K1L$^-$CP77$^-$ or K1L$^-$CP77$^+$ VV at a multiplicity of 30 PFU/cell and harvested at the indicated times after infection. Aliquots were used for protein determinations and spectrophotometric βGAL assays.

Viral protein synthesis in RK13 cells

For initial experiments, we took advantage of the late promoter-regulated *lacZ* gene to compare βGAL synthesis in RK13 cells infected with the recombinant viruses. In cells infected with K1L$^+$CP77$^-$ VV, βGAL activity was detected by 8 hr and progressively increased over a 24-hr period (Fig. 7). By contrast, βGAL activity remained at nearly background levels up to 24 hr after infection with K1L$^-$CP77$^-$ VV. When RK13 cells were infected with K1L$^-$CP77$^+$ VV, βGAL synthesis was considerably delayed and the activity at 24 hr was substantially less than that in cells infected with vK1L$^+$CP77$^-$ in accordance with the measurements of βGAL RNA in the previous section.

Metabolic pulse-labeling followed by SDS–PAGE was carried out to obtain a global view of protein synthesis in VV-infected cells. When RK13 cells were infected with the wild-type virus (K1L$^+$CP77$^-$), the expected pattern of viral polypeptide synthesis was observed by autoradiography (Fig. 8A). During the first few hours of infection, several bands increased in intensity, suggesting that they are viral early proteins. Viral late proteins were dominant from 3.5 hr on. In contrast, when RK13 cells were infected

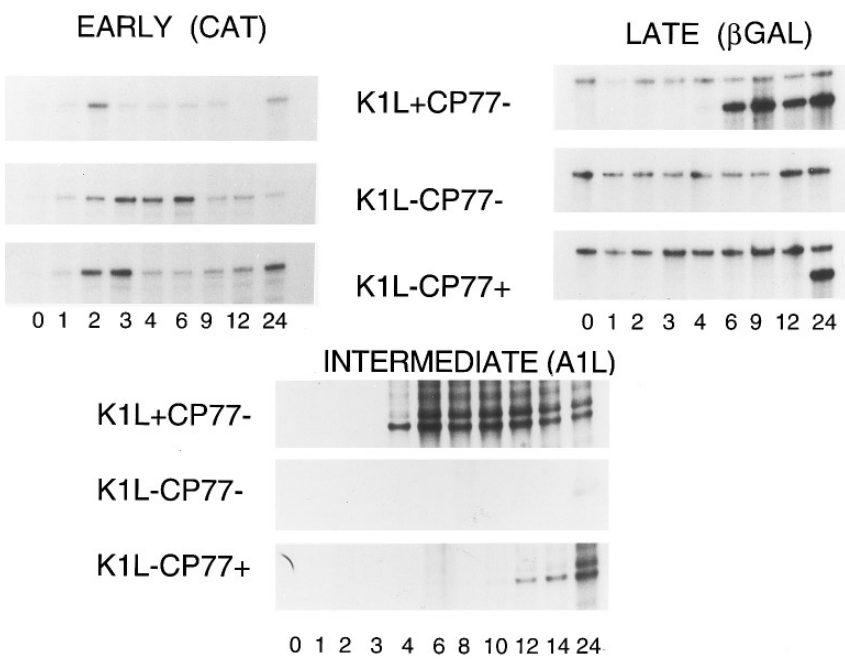

FIG. 6. Analysis of viral RNA levels. RK13 cells were infected with K1L$^+$CP77$^-$, K1L$^-$CP77$^-$, or K1L$^-$CP77$^+$ VV at a multiplicity of 30 PFU/cell. At the indicated hour after infection, the cells were harvested and samples prepared for nuclease protection analysis with ^{32}P-labeled early (CAT), intermediate (A1L), or late (βGAL) probes. Autoradiographs of the polyacrylamide gels are shown.

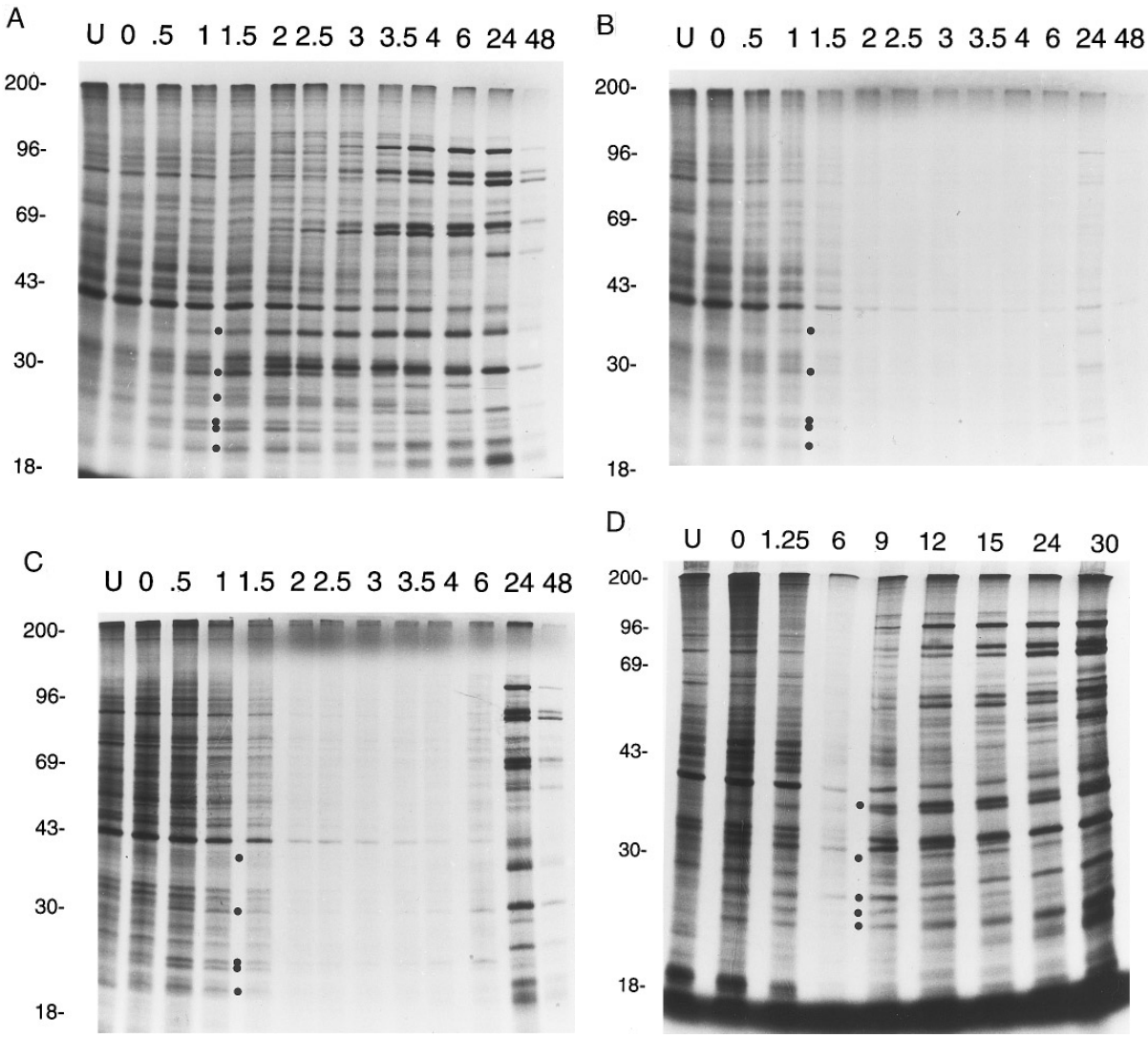

FIG. 8. Analysis of metabolically labeled viral proteins. RK13 cells were incubated with 30 PFU/cell of K1L$^+$CP77$^-$ (A), K1L$^-$CP77$^-$ (B), or K1L$^-$CP77$^+$ (C, D) for 30 min. At the end of this incubation period, the cells were pulse-labeled with [^{35}S]methionine for 30 min (0 time) and at the subsequent indicated hours after infection. The cells were lysed and radiolabeled proteins analyzed by SDS–PAGE. The positions of protein standards of indicated molecular mass (kDa) are indicated on the left of the autoradiogram. Putative early proteins are indicated by dots.

with either K1L$^-$CP77$^-$ VV (Fig. 8B) or K1L$^-$CP77$^+$ VV (Fig. 8C), a short period of early protein synthesis was followed by a dramatic reduction of viral and host protein synthesis, so that by 2 hr little metabolic labeling was evident. In RK13 cells infected with K1L$^-$CP77$^-$ VV, protein synthesis never recovered, whereas a remarkable recovery occurred by 24 hr in cells infected with K1L$^-$CP77$^+$. A closer examination of the recovery period revealed that protein synthesis resumed at about 9 hpi, at which time the pattern of bands was similar to that just prior to shutoff and appeared to include some early proteins (Fig. 8D). By 12 hr, the dominant late pattern of proteins was present.

Western blot analysis with rabbit antiserum against vaccinia virus was carried out to compare the accumulation of viral proteins synthesized in RK13 cells infected with the different recombinant VV and to rule out any labeling artifacts due to changes in amino acid transport or pool sizes. In addition, it is difficult to distinguish between cellular proteins and early viral proteins by metabolic labeling. With RK13 cells infected with wild-type (K1L$^+$CP77$^-$) VV, only early viral proteins were detected at 1 to 2 hr and late proteins accumulated between 4 and 24 hr (Fig. 9). In cells infected with K1L$^-$CP77$^+$ VV, the late protein pattern was established between 12 and 24 hr, whereas that pattern did not occur in cells infected with K1L$^-$CP77$^-$ VV (Fig. 9).

Recovery of viral early gene expression mediated by the CP77 gene product

The data thus far suggested that the absence of the K1L gene product imposed a premature arrest of transla-

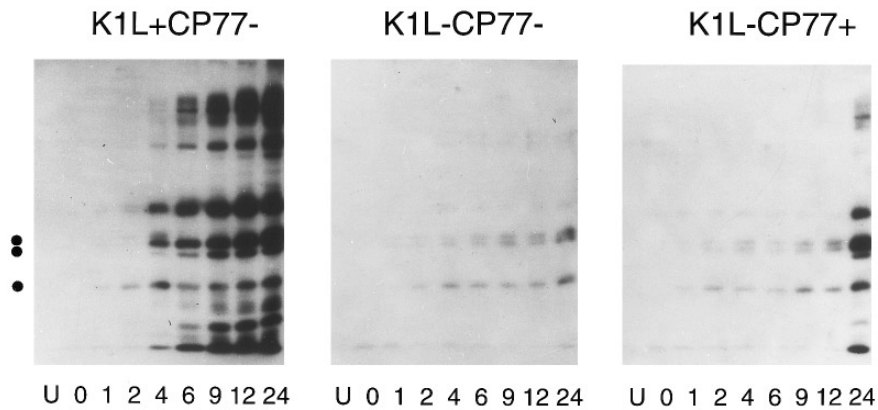

FIG. 9. Western blot analysis. RK13 cells were infected with K1L$^+$CP77$^-$, K1L$^-$CP77$^-$, or K1L$^-$CP77$^+$ VV at a multiplicity of 30 PFU/cell and harvested at the indicated hours after infection. Detergent lysates were electrophoretically resolved by SDS–PAGE, transferred to nitrocellulose membrane, and then incubated with anti-VV polyclonal antiserum at a 1:500 dilution. Antibody binding was visualized by autoradiography of the membranes after exposure to ^{125}I-protein A. Early proteins are indicated by dots.

tion of early mRNAs in RK13 cells. Since the arrest also occurred in cells infected with K1L$^-$CP77$^+$ VV, we considered the possiblity that the CP77 gene product overcomes RK13 host restriction by slowly facilitating DNA replication, thereby allowing intermediate and late gene transcription to proceed. Against this idea, however, was the suggestive resumption of viral early protein synthesis during the recovery period in RK13 cells infected with K1L$^-$CP77$^+$ VV seen in both pulse-labeling (Fig. 8) and Western blots (Fig. 9). If the latter were true, then we should more clearly see the recovery of viral early protein synthesis in the presence of AraC, an inhibitor of DNA replication. RK13 cells were infected in the presence of AraC and viral protein synthesis was analyzed by metabolic labeling and immunoprecipitation with rabbit antiserum against vaccinia virus (Fig. 10). Interestingly, the labeling pattern suggested the possibility of different kinetic classes of early proteins. Labeling immediately after the adsorption period (0 time) with wild-type (K1L$^+$CP77$^-$) VV resulted in the immunoprecipitation of several bands representing immediate early proteins. Synthesis of early proteins was maximal between 2 and 6 hr after infection in the absence of DNA replication and then continued at a lower level. By contrast, synthesis of early proteins was transient in cells infected with K1L$^-$CP77$^-$ or K1L$^-$CP77$^+$ VV and declined after 1 hr. However, with K1L$^-$CP77$^+$ VV, synthesis of the immediate early proteins resumed at 6 hr and increased between 10 and 24 hr. The completeness of the AraC block was suggested by the failure to immunoprecipitate three different intermediate proteins, A1L, A2L, and G8R (data not shown). We concluded from this experiment that the CP77 gene product is made in the presence of AraC and mediated the resumption of viral early protein synthesis independent of DNA replication.

DISCUSSION

Over the years, several investigators noted defects in protein synthesis during abortive replication of Orthopoxvirus host range mutants in a variety of cell lines (Brown and Moyer, 1983; Chen *et al.,* 1992; Drillien *et al.,* 1978; Moyer *et al.,* 1980; Njayou *et al.,* 1982; Sutter *et al.,* 1994). Evaluation of these results is difficult because of varied genetic backgrounds of the Orthopoxviruses, some having multiple deletions, and because many of the studies preceded present realization of the complexity of Orthopoxvirus gene regulation. In the most detailed analysis to date, we reported that in CHO cells infected with VV (strain WR) viral early mRNAs, early proteins, DNA, and intermediate mRNAs were made, but that synthesis of intermediate proteins, late mRNAs, and late proteins did not occur (Ramsey-Ewing and Moss, 1995). The presence of intermediate mRNAs but not intermediate proteins led us to consider a translational block. We have now carried out a similar analysis of the host-range defect in RK13 cells infected with a K1L deletion mutant of VV (strain WR). While metabolic labeling studies indicated a block in intermediate and late protein synthesis, as occurs in CHO cells, further experiments revealed an important difference. Host-range restriction in RK13 cells prevented viral DNA replication and synthesis of intermediate mRNAs. However, early mRNAs persisted despite the cessation of early protein synthesis, again suggesting a block in translation.

The different stages of host restriction in RK13 and CHO cells might be explained if early and intermediate mRNAs contained unique structural elements that serve as targets for translational inhibition. No early- or intermediate-specific sequences have been noted in either the coding or 3′-teminal sequences of VV mRNAs; moreover, reporter genes regulated by early or late promoters exhibit the same host-range expression defects as authentic VV mRNAs. There may, however, be a difference in the 5′ ends of most early and intermediate mRNAs. The three intermediate mRNAs examined by Baldick and Moss (Baldick and Moss, 1993) had a 5′ poly(A) leader, presumably formed by VV RNA polymerase slippage on

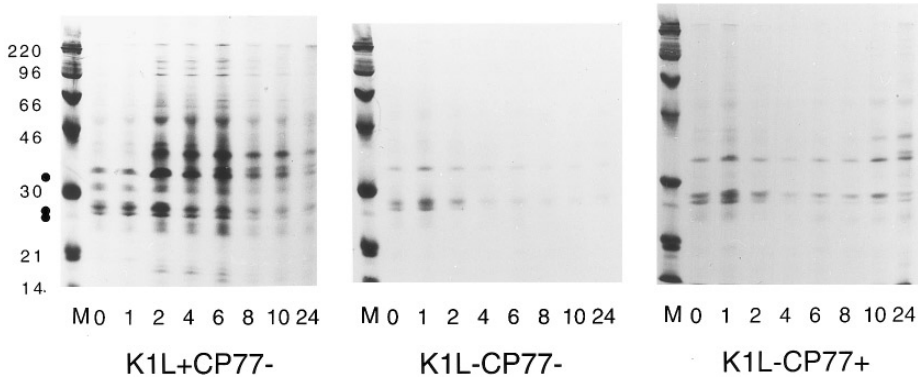

FIG. 10. Immunoprecipitation of metabolically labeled early viral proteins. RK13 cells were infected with K1L$^+$CP77$^-$, K1L$^-$CP77$^-$, or K1L$^-$CP77$^+$ VV at a multiplicity of 30 PFU/cell in the presence of AraC. At the indicated hour after infection, the cells were labeled with [^{35}S]methionine for 30 min and then lysed. Radiolabeled proteins were incubated with anti-VV polyclonal antiserum. Immune complexes were captured with Protein A–Sepharose beads. The bound proteins were washed, eluted by boiling in SDS sample buffer, and analyzed by SDS–PAGE. An autoradiogram is shown; early proteins are indicated by dots.

the TAAA sequence at the RNA start site, similar to that suggested for the poly(A) leader on late mRNAs (Davison and Moss, 1989b; Stunnenberg *et al.,* 1989). Although more intermediate mRNAs need to be analyzed, the feature will likely be a common one since the TAAA is an important promoter motif (Baldick and Moss, 1993). By contrast, only a few early mRNAs have a 5′ poly(A) leader (Ahn *et al.,* 1990; Ink and Pickup, 1990) and the TAAA is not an early promoter element (Davison and Moss, 1989a). The function of the the 5′ poly(A) leader has not been determined although a role in translational initiation has been suggested (Schnierle and Moss, 1992). An alternative possibility to explain the host-range differences is that early or intermediate mRNAs per se are not targeted but that the effects are manifested at early or intermediate times after infection. Thus, a translational defect at early times will effect early mRNA and a translational effect at intermediate times will effect intermediate mRNAs just because they are present.

The host-range defect in CHO cells can be overcome by inserting the cowpox virus CP77 gene into the *tk* locus of VV (strain Copenhagen) which entirely lacks the homologous gene (Spehner *et al.,* 1988) or by repairing the homologous gene of VV (strain WR) which has multiple frame-shifts (Ramsey-Ewing and Moss, 1995). Perkus *et al.* (1990) reported that the CP77 and K1L genes are functionally equivalent with regard to replication of the Copenhagen strain of VV in RK13 cells. We were especially curious about this result since the genes have no evident sequence similarity and our data indicated that the steps at which host restriction occurs in CHO cells and RK13 cells are different. If CP77 specifically enhances the translatability of intermediate stage mRNAs, then it would be difficult to understand how the block to translation of early mRNAs in RK13 cells is overcome.

Using recombinant VV derived from the WR strain, we confirmed the ability of the CP77 gene under its native promoter to permit replication of a K1L deletion mutant in RK13 cells. However, plaques formed in RK13 cells within 24 hr by K1L$^-$CP77$^+$ VV were much smaller than those formed by K1L$^+$CP77$^-$ and the virus yield also was considerably less. Despite this difference, K1L$^-$CP77$^+$ VV grew as well in BS-C-1 cells as wild-type virus and the yield of K1L$^-$CP77$^+$ VV was nearly 3 logs higher than that of wild-type virus in CHO cells. In addition, the sequence of a PCR copy of the CP77 gene, from K1L$^-$CP77$^+$ VV, was verified. Next, we compared the synthesis of viral DNA, RNA, and proteins in RK13 cells infected with wild-type (K1L$^+$CP77$^-$), K1L$^-$CP77$^-$, and K1L$^-$CP77$^+$ VV. For the first 6 to 12 hr after infection, the presence of the CP77 gene in the K1L deletion mutant had no detectable effect; cessation of viral and cellular protein synthesis occurred at the same time and to the same extent. Unfortunately, we have not yet obtained suitable antiserum to measure the synthesis of the CP77 gene product during this period. The first detectable effect of the CP77 gene was the resumption of early protein synthesis at 8 to 10 hr after infection. DNA replication occurred later, suggesting either that the genomic template had to be uncoated or that the replication machinery had not yet been made or assembled. The latter interpretation was supported by finding a similar delay in the replication of naked plasmid DNA transfected into RK13 cells infected with K1L$^-$CP77$^+$ VV. Following DNA replication, intermediate and late mRNAs and proteins were made.

Both pulse-labeling and Western blotting suggested that the remarkable resumption of protein synthesis in RK13 cells infected with K1L$^-$CP77$^+$ VV started with early species. This phenomenon was verified by carrying out the infection in the presence of an inhibitor of DNA replication. Under these conditions, synthesis of viral early proteins appeared to stop only to start again several hours later, consistent with a translational block that is eventually overcome even without DNA replication. The explanation as to why K1L permits

a smooth transition through the early, intermediate, and late stages of VV gene expression whereas protein synthesis is temporarily suspended when K1L is replaced by CP77 is most intriguing. The CP77 product may simply accumulate less rapidly or be less potent than the K1L gene product. These possibilities might be tested by increasing the level of CP77 expression. Alternatively, since K1L is neither needed for VV replication in CHO cells nor can compensate for CP77 in that system, the targets or actions of the two proteins may differ. In addition, both K1L and CP77 may have multiple roles as the former has been implicated in activating viral transcription (Rosales *et al.,* 1994) and the latter in preventing apoptosis (Ink *et al.,* 1995). It seems likely that these host-range proteins are interacting with cellular regulators of complex metabolic pathways and that further studies will be illuminating.

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